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PHARMACOLOGY

#949

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

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Application for Research Grant
(Use extra pages as needed)

Date: 11-15-73

1. Principal Investigator (give title and degrees):

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2. Institution & address:

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Nashville, Tennessee 37232

3. Department(s) where research will be done or collaboration provided:

Department of Pharmacology

4. Short title of study:

Influence of Nicotine on the Release of Acetylcholine in the Human Placenta and its Implications on the Fetal Growth

5. Proposed starting date: January 1, 1974

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

The existence of an acetylcholine (ACh)-choline acetyltransferase (ChA)*-acetylcholinesterase (AChE)**-like system in the human placenta has been demonstrated by a number of workers. Since the placentae of man and animals lack innervation, a number of questions arise about (a) the similarities and differences between ACh-ChE-AChE systems of nervous and non-nervous sources, (b) the significance of the location of cholinergic system in the human placenta, (c) the degree of permeability of the placental barrier to drugs and environmental chemicals and (d) the influence of the cholinergic system on the nutritional health of the fetus and the maintenance of pregnancy.

The ChA's from placental and nervous sources have been investigated in our laboratories and they were found to be similar in their chemical properties and the enzyme mechanism (1-3). According to present indication, ChA is located in the chorianic villi, in the cell layers separating maternal and fetal blood compartments (4). The major component of the ACh-like substances in the human

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*Recommended trivial name for Acetyl-CoA: choline O-acetyltransferase (2.3.1.6) by the International Union of Biochemistry. Other name: choline acetylase.

**Recommended trivial name for Acetylcholine hydrolase (3.1.1.7). Other names: true or specific cholinesterase.

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placenta has been identified as ACh by pyrolysis gas-chromatography (5). ACh was present in a bound form in the placenta. There is some experimental evidence that the bound ACh-like substances are located in "granules" which are liberated from the trophoblast epithelium (6).

In view of the high concentration and the location of ACh in the placenta, and the lack of any significant effect of ACh on the placental vascular system, (7-9) one is apt to suggest that the permeability of the placental barrier is controlled by the quantal release of ACh. If this statement were true, ACh-ChA-ChE system is of importance in the placental function during pregnancy. This placental function may be related to the transport of materials across the placental barrier, or release of placental hormones by ACh during pregnancy. A significant number of observations indicate the importance of the placental cholinergic system in the maintenance of pregnancy and the health of the fetus. Our results indicate that abortive human placenta contains higher levels of ACh than terminal placenta. Administration of anticholinesterase agents to pregnant mice causes the death of fetii (10). Ingestion of drugs with anticholinesterase activity as a side effect (e.g. morphine) induces fetal malformations in mice (11).

Cholinomimetics (e.g. pilocarpine) have been shown to exhibit anti-growth (or terotologic?) effects during chick morphogenesis (12). Some investigators have suggested that cholinomimetics produced the above conditions by affecting the hypothetical relationship between the cholinergic system and growth and morphogenesis (12) or by interfering with energy metabolism under in vivo conditions (13,14). However, there is no available evidence of the possible effect of cholinomimetics or quaternary ammonium compounds on the multi-step processes involved in the transport and metabolism of nutrients in vivo.

Recent surveys indicate that babies of women who are habituated to smoke tobacco are smaller in size than those of nonsmokers (15,16). Further recent studies indicate that nicotine releases ACh from presynaptic nerve terminals and synaptosomes (17,18). In view of these observations, one could anticipate that nicotine can release more than the normal quanta of ACh from its stores in the villous epithelium. Released ACh (and/or nicotine) may influence the transport of energy metabolites across the trophoblast. The final result would be the fetal deprivation and relatively poor fetal growth (Fig. 1). At the present time it is not possible to assess whether the final effect is due to released ACh or nicotine or both.

No studies are available on the effect of nicotine on ACh stores in placenta. Therefore, this application is made for evaluation of the following specific aims:

1. To study the effects of nicotine on the release of ACh from human placental villi and to establish relationships between the dose of nicotine and ACh released into the medium (or ACh retained in villi). The exact concentrations of ACh will be analyzed by pyrolysis gas chromatography.

2. To separate the ACh granules from placental tissue by density gradient separation, and to study the mechanisms of ACh release from granules by nicotine. The results in specific aim (1) lead to specific aim (2).

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7. Specific Research Aims (cont'd.)

3. To obtain evidence for the presence of a cholinergic receptor in the human trophoblast where nicotine binds for exhibiting agonistic or antagonistic effects. The results in specific aims (1) and (2) lead to specific aim (3).

4. To set up a perfusion system for human placenta for use in our laboratories. This system is useful for studying (a) the transport of drugs, energy metabolites or ions across the placental barrier during manipulation of placental cholinergic system by nicotine and other pharmacological agents, (b) the effects of nicotine on ACh release and transport of energy metabolites across placental barrier simultaneously, and (c) the effects of nicotine in human placenta collected at different gestation periods. Studies in specific aims (1) - (3) lead to studies in specific aim (4).

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8. Brief statement of working hypothesis:

The placenta plays an important role in the transport of materials between mother and fetus in a most complex way (Fig. 1). This role may be related to the active involvement of placenta in assisting and regulating transport of water soluble and ionizable materials. According to our present indications, ChA is located in the chorionic villi, in the cell layers separating maternal and fetal compartments. According to our investigations, ACh is located in chorionic villi in a bound form, possibly as granules whose contents may be released from the trophoblast epithelium. Free ACh may stimulate a cholinergic receptor on the trophoblast epithelium and thereby induce changes in its permeability to charged molecules. One would anticipate rhythmic fluctuations in the permeability of the trophoblast epithelium with constant shifting of the dominant site, which in turn may be dependent of the amount of ACh released. The changes in the permeability of the trophoblast epithelium facilitates the exposure of the various molecules on the maternal side to their corresponding carriers (or the transport processes). In other words, ACh stimulation of the cholinergic receptor in the trophoblast is coupled to the transport of substances across the placental barrier. This "ACh stimulation-transport coupling" is delicately balanced by the amount of ACh released. Both very low and high concentrations of free ACh are deleterious to the fetus.

According to the above hypothesis, nicotine may have two sites of action. Nicotine may mimic ACh and stimulate the cholinergic receptor in the trophoblast. Cholinomimetics are known for exhibiting antigrowth effects. Nicotine is also known for exhibiting antagonistic effects at high concentrations. Further, nicotine may release excessive quanta of ACh from its bound form. Excessive free ACh is known for exhibiting antagonistic effects at other types of cholinergic receptors. Therefore, the antigrowth effects of nicotine are possibly due to its antagonistic effects at the trophoblastic cholinergic receptor directly or through the excessive release of free ACh or both.

The various steps in the working hypothesis of ACh-stimulation and transport coupling are schematically represented in Fig. 2. The data, on which the above scheme is based, were obtained by a number of investigators using in vitro and in vivo experimental models of stimulation-secretion of biogenic amine stimulation-contraction by biogenic amines under a variety of conditions. Not all of these processes are based on firm evidence and some are only tentative. However, this schematic representation provides an outline for studying the sites of action of nicotine in the placenta and its effects on placental transport of nutrients.

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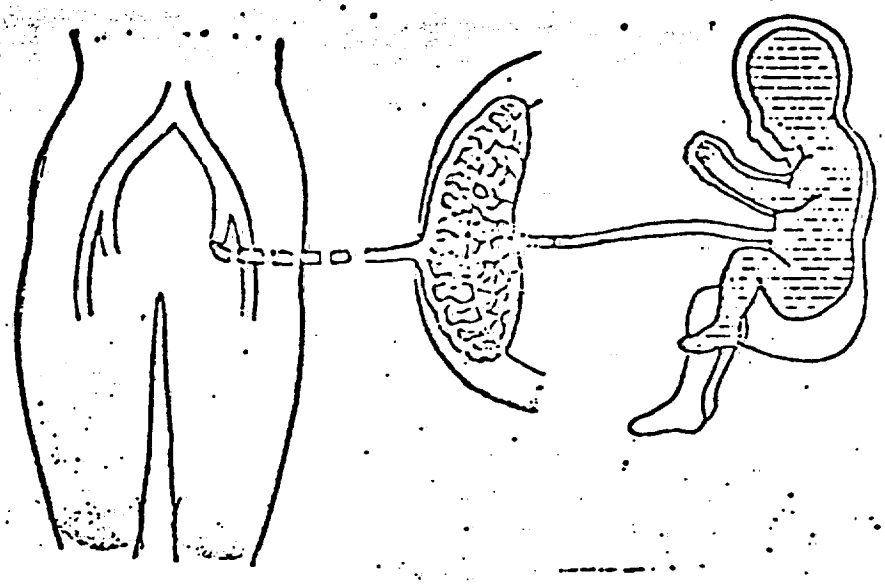
MATERNAL, PLACENTAL AND FETAL FACTORS WHICH MAY CAUSE POOR FETAL GROWTH:

POSSIBLE SITE OF ACTION OF NICOTINE



MATERNAL

↓

BLOOD

(a) Composition

(b) Circulation

(c) Foreign materials

PLACENTAL

(a) Pathologic lesions

(b) Physiologic limitations

↓

Permeability of placental barrier

FETAL

(a) Inability to grow for unknown reasons

(b) Due to limitations induced by maternal and placental factors

Nicotine in Maternal Blood

Release of ACh

↓

Modifications in Permeability of Nutrients

Limitations for Fetal Growth

Interaction at a Receptor

↓

Modifications in Permeability of Nutrients

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Figure 1

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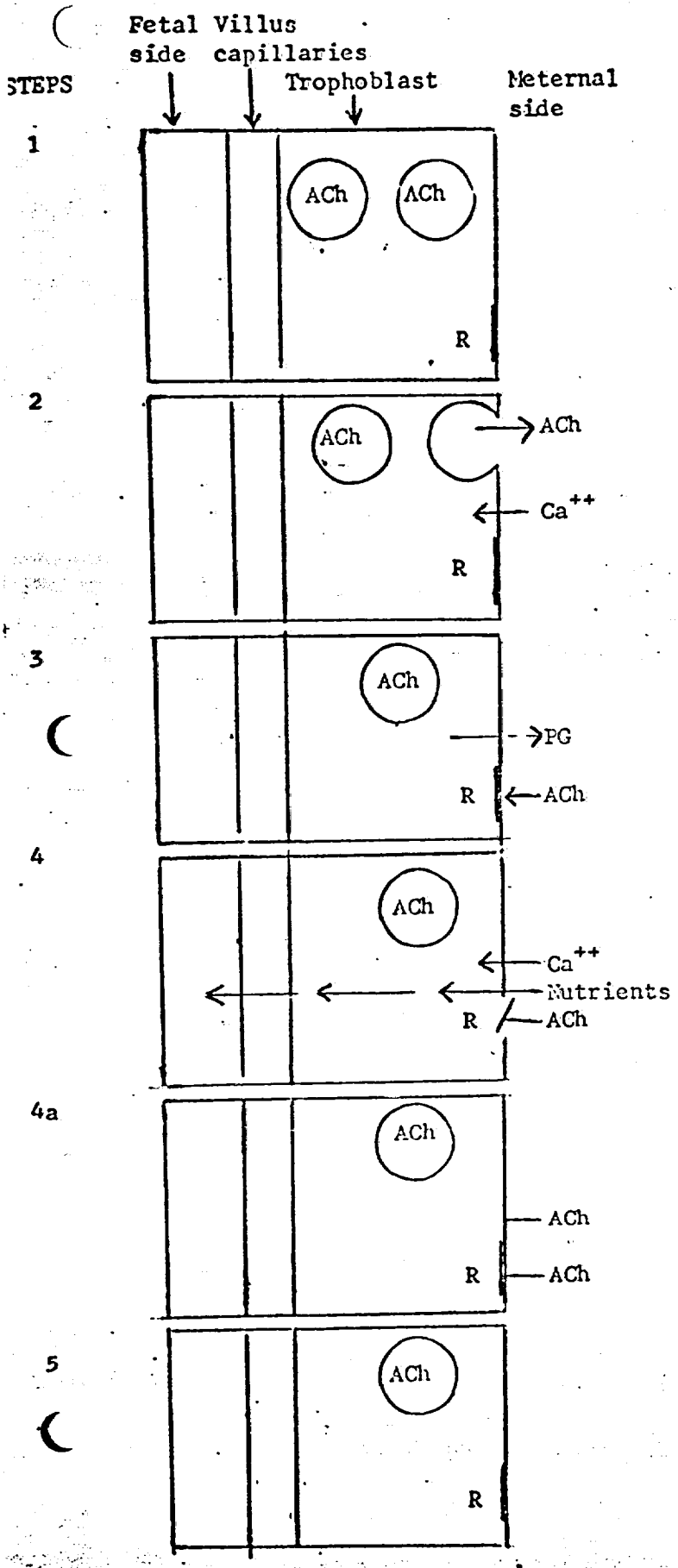
VARIOUS STEPS IN THE WORKING HYPOTHESIS OF ACh-STIMULATION AND TRANSPORT COUPLING IN THE TROPHOBLAST



Description of Steps

1 Brownian movement brings a ACh-granule to the inner side of the membrane

2 Available Ca^{++} or its influx facilitates the fusion of the granule with the membrane. The contents of the granule are released on the maternal side.

3 Membrane releases prostaglandins(PG), which nullifies the effect of Ca^{++} and restores the membrane. Released ACh interacts with the receptor (R)

4 R-ACh complex increases the permeability of the trophoblastic membrane. Entry of Ca^{++} and nutrients is facilitated. The nutrients from the trophoblast diffuse to the fetal side through the villus capillary walls
In low concentration, nicotine may mimic ACh

4a If excess ACh is released it interacts at nonspecific sites and nullifies the effect of ACh at R.
Nicotine may release excess ACh. It may block the receptor. The amounts of nutrients, which enter the trophoblast, may decrease due to the antagonistic effects of nicotine, ACh or both.

5 ACh effect is terminated due to its hydrolysis by AChE. Membrane stress due to depolarization leleases PG which nullifies Ca^{++} effect and resrores the membrane

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FIGURE 2

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9. Details of experimental design and procedures

Procedures for the collection of human placentae at different gestation periods:

The human placentae were collected from the Vanderbilt University Hospital delivery rooms. During the first trimester, the placentae were collected during therapeutic abortions. During the second trimester, the placentae were collected during spontaneous abortions or premature deliveries. During the third trimester, placentae were collected during natural or therapeutic deliveries. Each placenta after delivery was placed in a plastic bag immediately after delivery and was cooled in an ice bath. The fresh placenta was processed immediately for (1) ACh analysis, (2) experiments involving the perfusion of placental lobes and (3) uptake and release of nutrients, ions and drugs by placental slices and isolated villi. Some placental samples were frozen at -12°C for future analysis of AChE and ChA.

Experimental procedures to study the release of ACh from isolated villi:

It is very easy to tease out villi of the human placenta, providing a considerably less traumatized preparation for in vitro study than the placental slice (19). The trophoblast is exposed to the incubation medium as much as it is to the maternal circulation under normal conditions. We have successfully used such preparations to study the distribution of ACh in human placenta. Such preparations have survived for 6 hours in our laboratories in phosphate or bicarbonate buffers. We have done some initial experiments for the release of ACh into the medium as a factor of time. Our studies on the effects of excess K+, Ca++, cyclic AMP, cyclic GMP and prostaglandins on the release of ACh are in progress. We propose to study the effects of nicotine on the release of ACh in the same system.

The isolated villi were incubated for different time periods in an incubation medium (phosphate or bicarbonate buffer) at 37°. The amounts of ACh in the medium and the villi were determined. In a second series of experiments various doses of nicotine (10^{-6} to 10^{-4}M) were added to the medium and again ACh concentrations were determined. From these experiments the net release of ACh into the medium was calculated.

In our preliminary experiments, it was found that ACh was continuously synthesized by ChA and released into the medium. In a third series of experiments, the medium contained a ChA-inhibitor (halogeno-acetylcholines) for preventing the synthesis of new ACh. In a fourth set of experiments the medium contained a ChA inhibitor as well as nicotine. From these two sets of experiments the effect of nicotine on the preformed ACh could be calculated.

In all experiments, the medium contained physostigmine (10^{-6}M) for inhibiting the hydrolysis of ACh by placental AChE.

Experimental procedures for studying effects of nicotine on the release of ACh from ACh-granules of human placenta:

We have indirect evidence that 95% of total human placental ACh is in a bound granular form in human placenta. We propose to isolate these granules using techniques of subcellular fractionation described by De Robertis (20) and Whittaker (21). We propose to study the release of granular ACh using various techniques and treatments described by Whittaker and his collaborators (22,23).

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9. Details of experimental design and procedures (cont'd.)

We have used a second approach for the separation ACh-granules from placenta. The trophoblast was separated easily from the trophoblast by dissection. Then the cut trophoblast was subjected to digestion with trypsin and collagenase. Then the contents were subjected to subcellular fractionation, and obtained a rich fraction of ACh-granules. We propose to study the effect of nicotine on these granules.

Experimental procedures for characterization of cholinergic receptors (nicotinic-type or muscarinic type) in placental syntrophoblast:

The working hypothesis assumes the presence of a cholinergic receptor with which ACh or nicotine interacts. Therefore, evidence for the presence of a cholinergic receptor will be obtained.

The binding constants of ^{14}C-ACh and ^{14}C-nicotine to the cholinergic receptors will be determined using the techniques of centrifugal assay and equilibrium dialysis developed by O'Brien and his collaborators (24,25).

The receptor will be characterized as muscarinic or nicotinic by determining the binding constants of specific ^{14}C-labelled agonists and antagonists. Specific agonists: acetyl-l-^{14}C-β-methylcholine for muscarinic receptors, ^{14}C-nicotine for nicotinic receptors. Specific antagonists: ^{14}C-atropine for muscarinic receptors, ^{14}C-d-tubocurarine or ^{14}C-decamethonium for nicotinic receptors. In receptor protection experiments, the unlabelled agonist should prevent the binding of the labelled antagonist to the receptor. The binding of ^{14}C-labelled agonists and antagonists to the receptors will be verified by the radioautographic procedures described by Waser and his collaborators (26). The binding constants of various agonists and antagonists to cholinergic receptors in tissues other than placenta were determined in the author's laboratory (27,28), with which the binding constants of the cholinergic receptors in human placenta will be compared.

If the human placenta is shown to contain cholinergic receptors, it will become the easily available human source for the isolation of cholinergic receptors. So far cholinergic receptors were not isolated from any mammalian source.

Experimental procedures for studying the transport of nutrients across the placental barrier under the influence of nicotine: perfusion of human placenta:

A method for perfusing a single lobe of human placenta was developed by Schneider et al. (29). This system will be used in the present experiments. In this setup, an effective maternal, as well as fetal, circulation was established. Therefore, this system is useful for transport studies. The experimental details and techniques of placental perfusions were described by Schneider et al. (29).

The fetal vessels were cannulated and perfusion of fetal circuit was started at a flow rate of 6-12 ml/min at 50-90 mm Hg. Flow rates were checked to make certain that arterial inflow equals venous outflow. This confirmed that an isolated vascular unit has been cannulated and that there was no gross leak across placenta.

Two glass cannulas delivering the maternal perfusion fluid were introduced into the intervillous space through the basal plate. The intervillous space was perfused at a rate of 10-15 ml/min at 80-140 mm Hg. The maternal perfusate

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9. Details of experimental design and procedures (cont'd.)

returned from the intervillous space through multiple venous openings in the basal plate and was collected for analysis.

The perfusate was Earle's buffered salt solution containing 4 g% of dextran (low molecular weight) and 100 mg% of glucose. The colloidal osmotic pressure of the perfusate was equivalent to that of normal plasma. The fetal perfusate was equilibrated against a gaseous mixture containing 5% O_2, 5% CO_2 and 90% N_2. The maternal perfusate was equilibrated with 95% O_2 and 5% CO_2. The perfusates were not recycled.

The nutrients, whose placental transport was measured, were added to the maternal circulation and their clearances from the maternal to fetal circulation were measured. The clearances were calculated as follows:

$$\text{clearance (ml/min)} = \frac{(FV-FA)FFR}{MA}$$

where FV = concentration in fetal venous outflow (mg/ml)

FA = concentration in fetal arterial inflow (mg/ml)

MA = concentration in maternal arterial flow (mg/ml)

FFR = fetal flow rate (ml/min)

A series of clearances of a nutrient can be measured before and after addition of various doses of nicotine to the maternal circulation. Using this basic perfusion system, it is possible to study the effects of (a) nicotine, (b) nicotine in the presence of a cholinesterase inhibitor for preventing the enzymatic hydrolysis of ACh which is released by ACh, and (c) nicotine in the presence of a ChA-inhibitor for preventing de novo synthesis of ACh on the clearance of selected ions, amino acids, sugars and proteins across the placental barrier.

The following agents are suitable as tools for studying the effects of nicotine on placental transport:

Cholinesterase inhibitors: Physostigmine, neostigmine

ChA-inhibitors: Halogeno-acetylcholine (Iodo-bromo- and chloro-acetylcholines)
N-methyl-4(-1-naphthyl-vinyl) pyridine

Ions: K^+, Na^+, Ca^{++}

Amino acids: ^{14}C-α-aminoisobutyric acid

Proteins: ^{131}I-labelled gamma globulin

Sugars: D-xylose, L-xylose

The specific cholinesterase inhibitors specified above were selected for this study, because their pharmacological effects on cholinergic system, other than that of placenta were well documented. Halogenoacetylcholines have been synthesized by the principal investigator and his collaborators and their pharma-

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9. Details of experimental design and procedures (cont'd.)

cology is known (1-3,30-36). N-methyl-4(-1-naphthyl-vinyl)pyridine and its analogs have been introduced by Cavallito and their collaborators as choline acetylase inhibitors (37) and these compounds are available for this investigation from commercial sources.

The ions, K+, Na+, Ca++, were selected for this study due to the following reasons: The placenta is very effective to protect the fetus against potassium depletion (19,38). The reasons for this observation are not known. The maternal hypokalemia is not reflected in the fetal blood or tissues. With sodium deprivation of the mother, various degrees of hyponatremia are induced in the fetus of the rat or the sheep (39,40). The results are variable and are not conclusive as to whether the placenta protects the fetus against sodium deprivation (19,40). In human placenta, there is a relationship between ^{24}Na transfer and gestational age (41). The transfer rate increases until 36 weeks of gestational age and then decreases at term. A fall off in placental efficiency towards term is consistent with the clinical concept of post-maturity (38). In our experiments fluxes of ^{24}Na and variations in ACh concentrations will be measured simultaneously within the same placental isolated villi. Ca++ is included in this because of its importance to fetal growth and its possible role in the secretion of ACh.

Placenta concentrates ^{14}C-α-aminoisobutyric acid from maternal circulation against a concentration gradient and releases on a downhill gradient to fetus (42). As this amino acid is non-metabolizable, it is suitable to study the effects of cholinergic agents on the placental transfer of amino acids. It is of interest to note here that a cholinergic mechanism has been implicated as a fine control in regulating the production of protein in spiders (43).

One of the factors in the protection of the newborn is the antibodies received passively from the mother, and the mechanisms for this transfer are not known. The investigations of Dancis et al. (44) indicate that the route for the transfer of proteins from mother to fetus is allantoic placenta. This transfer of proteins is significant in the early pregnancy and in late pregnancy in the rat (45), monkey (46), and human (44). In the above studies or protein transfer ^{131}I-labelled gamma globulin has been used. In the experiments of the above design the protein-bound radioactivity represents transferred protein (47). Therefore, ^{131}I-labelled gamma globulin will be used to measure placental transfer of proteins during manipulation of placental cholinergic system by nicotine.

D-xylose, which is transferred through the placenta at 3 times the rate of L-xylose, is a nonmetabolizable model for glucose. Therefore, the placental transfer of D-xylose will be measured during the manipulation of cholinergic system by nicotine (19,48).

Analytical Methods

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Assay of ACh in placental tissues by gas chromatography:

A representative sample of placenta is cleared of blood clots, and is homogenized in a Sorvall Omnimixer containing acetonitrile and trichloroacetic acid (1.0 ml/0.1 g wet tissue, TCA 2%). The homogenate was centrifuged and the precipitate was rejected. The supernatant was diluted with equal volume of distilled water and the resulting solution was extracted twice with equal volumes of diethyl ether and the ether layer was discarded. A gentle stream of nitrogen gas was

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bubbled through the aqueous layer to remove the residual ether. The ether-free aqueous layer (2.0 ml) was diluted with equal volume of distilled water. To the resulting solution tetramethylammonium iodide (6.0 μg) and KI-I_2 solution (0.15 ml) were added. The reaction mixture was centrifuged and the supernatant was aspirated. The precipitate of quaternary ammonium compounds was subjected to gas chromatography using pyrolysis techniques (49,50) or Jenden's reaction (51,52).

This method is schematically represented in Figure 3.

Analysis of tissues for cholinesterases:

Three different types of methods are routinely used in our laboratories for the assay of cholinesterases. In all three methods ACh was used to give total cholinesterases. Acetyl-β-methylcholine was used as a specific substrate for AChE because it was not hydrolyzed by pseudo-ChE (53). Butyrylcholine was used as a substrate for the analysis of pseudo-ChE (54). It is a very weak substrate of AChE. However, a correction factor has been developed for the hydrolysis of butyrylcholine by AChE for the assay of pseudo-ChE (55). Each method is applicable for a specific situation based upon the amount of tissue available and the sensitivity required in the assay. In all three methods tissue homogenates were used as the source of the enzymes.

In the first method, the hydrolysis of choline esters were determined by titrating the acids released during their hydrolysis using a Radiometer pH-stat titration unit. The details were described in the papers published in the literature (56).

The second method is a differential radiochemical assay for AChE and pseudo-ChE using ^{14}C-substrates (55). The differential rates of hydrolysis of 1-^{14}C-acetyl-β-methylcholine (pS 2.5) and 1-^{14}C-butyrylcholine (pS 2.5) were used to estimate AChE and pseudo-ChE activities in tissues. The tissue homogenate and the ^{14}C-substrate were incubated for varying periods (0-50 min). Initial linear velocities were obtained from rates of hydrolysis during 0-10 min. Acetyl-β-methylcholine was not significantly hydrolyzed by pseudo-ChE, while butyrylcholine was hydrolyzed by AChE at 12% of the rate of acetyl-β-methylcholine. Therefore, a correction was applied in the estimation of pseudo-ChE by butyrylcholine. This method is of special value in estimating cholinesterases in small amounts of tissues (plasma 0.01-0.1 ml, tissue, 10 mg). This method has been used successfully for the estimation of cholinesterases in the human placenta.

In a third method, $^{14}CO_2$ produced from the interaction between $NaH^{14}CO_3$ and acid products of esters hydrolyzed by cholinesterases were trapped with 2-phenethylamine and counted with a liquid scintillation counter.

Experiments were done using a Warburg apparatus with 0.4 ml of 2-phenethylamine placed in the central well of the flask to trap $^{14}CO_2$ produced. The main compartment of the 15 ml Warburg flask contained 2.5 ml of modified Krebs-Ringer bicarbonate buffer with 1/10 the normal concentration of $NaH^{14}CO_3$ and a radioactivity level of 2.22×10^5 dpm (2.3×10^{-3} M $NaH^{14}CO_3$, 7.5×10^{-2} M KCl, 7.5×10^{-2} M NaCl, 4×10^{-2} M $MgCl_2$). Tissue samples containing the enzyme (0.2 ml) were added to the main compartment of the flask. The side arm contained 0.3 ml of substrate (final concentration of 10^{-3}M) dissolved in the modified buffer which contained no radioactivity. The Warburg flasks were shaken for 15 minutes before the substrate (ACh, acetyl-β-methylcholine, or butyrylcholine) was dumped

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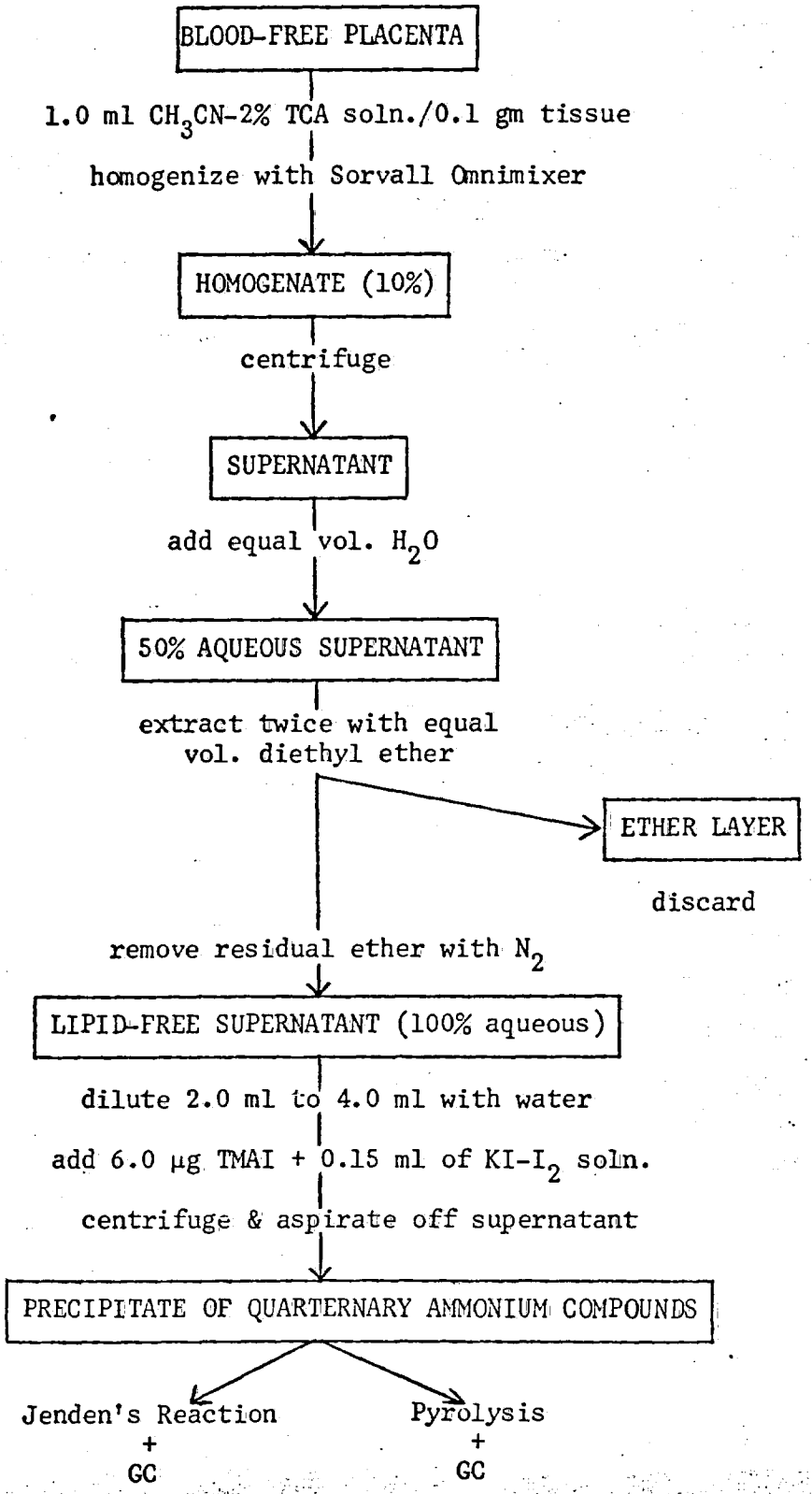

Figure 3: The 5 step procedure includes: (a) Extraction with CH_3CN and trichloroacetic acid, (b) Centrifugation to remove insoluble membranes, (c) Removal of lipids with ether, (d) Precipitation of quaternary ammonium compounds with potassium iodide, iodine and tetramethylammonium iodide, (e) Subjection of quaternary ammonium compounds to pyrolysis or Jenden's reaction.

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9. Details of experimental design and procedures (cont'd.)

into the main compartment. The reaction mixture was shaken for various periods of time and the 2-phenethylamine with $^{14}CO_2$ was transferred to a counting vial containing 10 ml of scintillation cocktail (2,5-diphenyloxazole, 7 g.; napthalene, 100 g.; dioxane, 1000 ml). The central well was washed twice with 2 x 0.3 ml of methanol and the washings were added to the scintillation cocktail and counted with a liquid scintillation counter. Further details of the method were described by Sastry and White (53) and Sastry and Chiou (57). This method was very useful to determine cholinesterases bound to membrane fractions.

Analysis of choline acetyltransferase (ChA) placental tissues:

ChA in placental tissues was assayed by the formation of acetyl-^{14}C-choline from choline and acetyl-^{14}C-CoA. The details for the separation of ^{14}C-ACh from the reaction mixture and counting of the samples were described in the papers by the author and his collaborators (1-3), and McCamen and Hunt (58). We used this method in the purification of ChA from the human placenta (1,2) and the rat brain (3).

Analytical methods for important ions: Conventional analytical methods with necessary modifications will be adopted. The following methods were listed as examples:

(a) Sodium and potassium: Emission flame spectrophotometry
(b) Chloride: Automatic chloride titrator (59)
(c) Calcium: A micromethod developed by Geyer and Bowie (60) is routinely used in the author's laboratory to determine the concentrations of calcium in biological samples. The method is based on emission flame spectrophotometric techniques. The final samples contain isopropyl alcohol, water and known concentrations of sodium and potassium chlorides. Isopropyl alcohol is used to increase the luminosity. Sodium and potassium chlorides are employed to control the normal interference from sodium and potassium. Samples are analyzed employing oxygen-hydrogen flame at a wave length of 422.66 mμ with a slit width of 0.04 mμ. The red filter is employed in conjunction with the photomultiplier. Calcium values less than 2 μg/ml are readily analyzed by this method. The author's laboratory contains a Beckman DU-2 spectrophotometer equipped with flame attachment, spectral energy recording adaptor and a recorder.

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Radiometric methods:

The isotope ions, ^{24}Na and ^{42}K, used in the present investigation can be measured by gamma scintillation spectrometry using a multi-channel analyzer. ^{45}Ca will be assayed by beta counting methods. Gamma scintillation spectrometric methods have been used in authors laboratory during the last 10 years, and typical examples are described in author's papers (61,62).

^{14}C-compounds will be assayed using liquid scintillation methods. Typical examples are described in author's papers (1-3) and the papers by Harbison and his collaborators (63,64).

Source: https://www.industrydocuments.ucsf.edu/docs/tjll0000

10. Space and facilities available

Total space (2 laboratories, 1 office, 1 instrument room) available for Dr. Sastry consists of 936 square feet, equipped with modern conventional laboratory facilities suitable for chemical, radio-biological and animal work. These facilities contain a roomette (which has separate vents for removal of odors) for animal surgery and isolation. These laboratories contain built in refrigerators (3), radiation hoods (2), and a walk-in hood for chemical syntheses.

The human placentae were collected from the delivery rooms and surgical facilities of Vanderbilt University Hospital with the help of the staff of the Department of Obstetrics and Gynecology. The Department of Pharmacology, the Department of Obstetrics and Gynecology, and the delivery and surgical rooms of Vanderbilt University Hospital are located within the same building complex. The collection and transport of placenta after delivery takes less than 5 minutes from the delivery or surgical rooms to the Department of Pharmacology.

A number of items of equipment are available in the author's laboratory to perform unit processes for conducting various steps in this research.

1) For chemical and physical manipulations:

a) Fisher-roll around vacuum system
b) Bendix Automatic Polarimeter
c) Precision Scientific-Lotemptrol Circulating Unit
d) Welsh vacuum pump

2) For chromatographic identification:

a) Misco-Electrochromatography Unit
b) Precision-Scientific-Chromac for circular paper chromatography
c) Equipment for ascending, descending and circular paper chromatography

3) For analytical procedures:

a) Aminco-respiration apparatus with accessories
b) Radiometer pH-stat titration unit equipped with a pH meter, a recorder and an autoburette
c) Beckman DU-Spectrophotometer (with flame attachment)
d) Corning Model-12 Research pH Meter
e) Nuclear-Chicago Autogamma system with a 512 channel analyzer
f) Metler-semimicro and macrobalances
g) Cahn Electrobalance (capacity, 1000 μg)

4) For bioassay procedures:

a) Harvard Kymograph
b) Gilson Polygraph and accessories (4 channels)
c) Narco-Biosystems Physiograph-desk model DMP 4A (4 channels)
d) Isometric and isotonic transducers (4 each)
e) Phipps & Bird-Isolated organ tissue baths (2)
f) Grass 88 stimulator
g) Harvard continuous infusion and peristaltic pumps
h) Harvard respirators for dogs, rats and other small animals

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Source: https://www.industrydocuments.ucsf.edu/docs/tjll0000

10. Space and facilities available (cont'd.)

5) For statistics:

Marchant electronic calculators, Cogito 516 & 1016 PR

6) Equipment in the Department of Pharmacology available for part time for this project:

a) Beckman Model L Ultracentrifuge
b) Sorvall-Superspeed Automatic Refrigerated Centrifuge
c) Beckman DK Recording Spectrophotometer
d) Farrand Spectrofluorometer
e) Packard Tricarb Liquid Scintillation Spectrometer (Model 3375)
f) Olivetti Underwood Programmer, Model 101
g) Nuclear Chicago 512-Channel Gamma Scintillation Spectrometer equipped with multiregion-interest-accessory, sample changer (capacity) and teletype
h) Hewlett-Packard Model 5750 gas chromatograph

11. Additional facilities required: None

12. Biographical sketches of investigator(s) and other professional personnel: Attached Separately

13. Publications: (five most recent and pertinent of investigator(s)):

1) Molecular Aspects of the Interactions of Halogeno-acetylcholines with Cholinesterase, B.V. Rama Sastry and Chung Y. Chiou, Biochim. Biophy. Acta, 167, 339-354, 1968.

2) Cholinergic activities of halogeno-acetylcholines. Chung Y. Chiou and B.V. Rama Sastry, J. Pharmacol. Exptl. Therap. 172: (2) 351-366, 1970.

3) Dissociation constants of D and L -lactoylcholines and related compounds at cholinergic receptors. H.C. Cheng and B.V. Rama Sastry, J. Pharmacol. Exptl. Therap., 180: 326-339, 1972.

4) Kinetic mechanisms of human placental choline acetyltransferase. B.V. Rama Sastry and G.I. Henderson, Biochem. Pharmacol. 21: 787-802, 1972.

5) Stereoisomerism and drug action in the nervous system. B.V. Rama Sastry (Invited Author), Ann. Rev. Pharmacol. 13: 253-267, 1973.

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Source: https://www.industrydocuments.ucsf.edu/docs/tjfl0000

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
B.V. Rama Sastry, Ph.D., Professor of Pharmacology	40%	$ --0--
Technical		
Lillian K. Owens, Research Assistant	100%	$ 7,878
Fringe Benefits		1,123
	Sub-Total for A	$ 9,001
B. Consumable supplies (by major categories)		
Chemicals and Drugs		$ 500
Glassware and Plasticware		500
Surgical Supplies		100
	Sub-Total for B	$ 1,100
C. Other expenses (itemize)		
Travel: To attend national meeting to present work supported by this grant		$ 300
Other: Expenses involved with obtaining human placenta		300
	Sub-Total for C	$ 600
	Running Total of A + B + C	$ 10,701
D. Permanent equipment (itemize)		
Minor items of equipment related to the gas chromatographic analysis of acetylcholine		$ 500
	Sub-Total for D	$ 500
E. Indirect costs (15% of A+B+C)	E	$ 1,605
	Total request	$ 12,806

C.K.
R.C.H.

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15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	$9,457	$1,100	$700	$-0-	$1,689	$12,946
Year 3	$9,977	$1,200	$700	$-0-	$1,782	$13,659

Source: https://www.industrydocuments.ucsf.edu/docs/tjll0000

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
"Biological Significance of Choline Esters Other than ACH"	NIH-NS04699	$42,544 44,822 47,303	9/1/73-8/31/74 9/1/74-8/31/75 9/1/75-8/31/76

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
"Cholinergic System, Placental Transport and Fetal Growth"	NIH HD08561	$311,746	5/1/74-4/30/79

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name B.V. Rama Sastry, D.Sc., Ph.D.

Signature B. V. Rama Sastry Date Nov 20, 1973

Telephone 615 (Area Code) 322-2207 (Number) N/A (Extension)

Checks payable to

Vanderbilt University

Mailing address for checks Mr. Paul Gazzerro, Jr.,
Associate Vice Chancellor for Medical Affairs
Operations and Fiscal Planning
Vanderbilt University Medical Center
Nashville, Tennessee 37232

Responsible officer of institution

Typed Name Allan D. Bass, M.D.

Title Associate Dean, Biomedical Sciences

Signature X Allan D. Bass Date 11/26/73

Telephone 615 (Area Code) 322-2281 (Number) N/A (Extension)

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